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Characterization of antigens recognized by monoclonal and polyclonal antibodies directed against uvomorulin

(cell adhesion/teratocarcinoma/membrane proteins)

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ABSTRACT **Uvomorulin is a cell surface glycoprotein involved in compaction of early mouse embryo. Antibodies, either monoclonal or polyclonal, raised against a purified tryptic fragment of uvomorulin recognize, in a detergent lysate of embryonal carcinoma cells metabolically labeled with ^{35}S, three molecules (120, 100, and 88 kDa) that are not related, as judged by peptide mapping. Only the 120-kDa form is related to the tryptic fragment of uvomorulin and, thus, is considered as the native form of uvomorulin. Although all three products are apparently detectable at the cell surface, only the 120-kDa form is glycosylated. Coimmunoprecipitation of the three different polypeptides is probably due to shared epitopes rather than to their presence in a multimeric complex.**

Uvomorulin (UM) was first described as a target of antisera raised against embryonal carcinoma (EC) cells because of its ability to inhibit their decompacting activity on early mouse embryos or EC cells (1). UM is a calcium-dependent cell-adhesion molecule that apparently has a wide tissue distribution (2–4). It is thought to be homologous to cell adhesion molecules described in other species (5–8). A rat monoclonal antibody (DE1) was prepared against a tryptic fragment of UM (UMt) (9). DE1 recognizes in the presence of calcium a series of products having different molecular masses (120, 100, and 88 kDa) in lysates obtained from radiolabeled EC cells (2). Because UM was first characterized on the basis of the functional activity of its tryptic fragment UMt, described as an 84-kDa glycoprotein (10), the identity of native UM can be established from a structural comparison with this polypeptide.

MATERIALS AND METHODS

Cells. Mouse EC lines PCC4 Aza R1 (11) and F9 (12) were used.

Labeling Procedures. For continuous labeling experiments, cells were preincubated for 1 hr in methionine-free Eagle's normal medium supplemented with 7% fetal calf serum and incubated 12 hr in the same medium with 100 μCi of [^{35}S]methionine (1350 Ci/mmol; Amersham; 1 Ci = 37 GBq) per ml.

For pulse–chase experiments, a similar procedure was used, but 250 μCi of [^{35}S]methionine was added per ml for 10 min. Cells were washed with Eagle's normal medium supplemented with 10% fetal calf serum and incubated in this medium (zero time of chase). Cells were lysed at the times indicated in the figures. For sugar-labeling experiments, cells were preincubated 1 hr in Eagle's normal medium containing 10% of the normal amount of glucose and 10% fetal calf serum and were incubated 18 hr in the same medium containing 100 μCi of D-[2-^3H]mannose, D-[1-^3H]galactose, or L-[2,3-^3H]fucose per ml or 50 μCi of D-[6-^3H]glucosamine hydrochloride (Amersham) per ml.

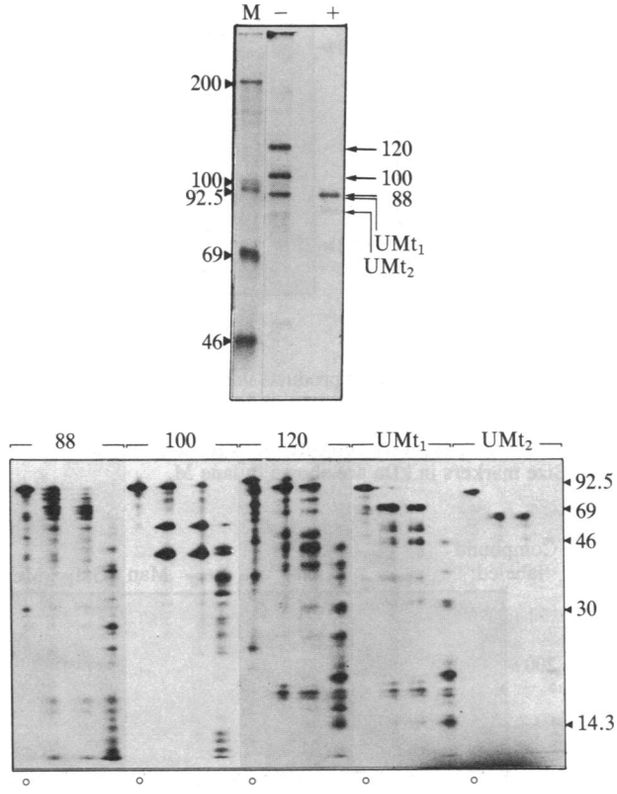

FIG. 1. Peptide mapping of UMt and products immunoprecipitated by DE1 from a detergent lysate of radiolabeled F9 cells. (*Upper*) Detergent extracts of cells were subjected to immunoprecipitation, and the immunoprecipitates were analyzed on a 7.5% $NaDodSO_4$/polyacrylamide gel. Lanes: −, detergent lysate immunoprecipitated in the presence of 2 mM $CaCl_2$; +, detergent lysate treated with trypsin and immunoprecipitated in the presence of 2 mM $CaCl_2$; M, Size markers (Amersham) indicated in kDa. (*Lower*) Products, detected without fluorography, were subjected to digestion with four different concentrations of protease: 25, 2, 1, and 0 ng (○) per sample (from the right to the left on the figure). UMt_1 and UMt_2 designate the fragments recognized by DE1 in a trypsin-treated detergent lysate of radiolabeled F9 cells (see lane − in *Upper*). They were described as 86- and 82-kDa products (2) and are thought to be virtually identical.

Inhibitors. The inhibitors of trimming glycosidases 1-deoxynojirimycin and 1-deoxymannojirimycin were present in preincubation, labeling, and chase media at a final concentration of 2 mM and 1 mM, respectively (13).

Cells labeled with [^{35}S]methionine in the presence of tunicamycin were preincubated 0.5 hr in Eagle's normal

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Abbreviations: UM, uvomorulin; UMt, tryptic fragment of uvomorulin; EC, embryonal carcinoma.

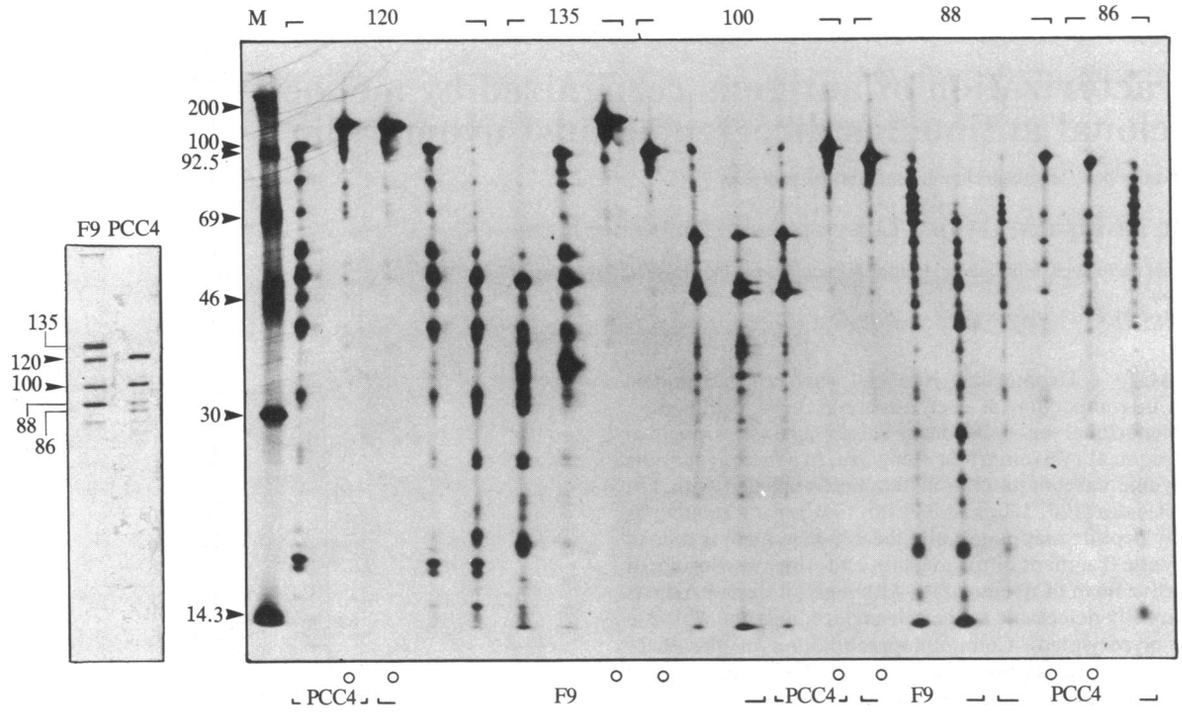

FIG. 2. Peptide mapping of products immunoprecipitated by DE1 from a detergent lysate of pulse-labeled F9 cells. (*Left*) Detergent extracts of cells were subjected to immunoprecipitation, and the immunoprecipitates were analyzed on 7.5% $NaDodSO_4$/polyacrylamide gel. Lanes: F9, detergent lysate of F9 cells pulse-labeled for 1 hr; PCC4, detergent lysate of PCC4 Aza R1 cells continuously labeled. (*Right*) Products, detected without fluorography, were subjected to digestion with 2 or 0 ng (○) of protease V8 (PCC4 Aza R1) or with 10, 2, or 0 ng (○) of protease (F9 cells). Size markers in kDa are shown in lane M.

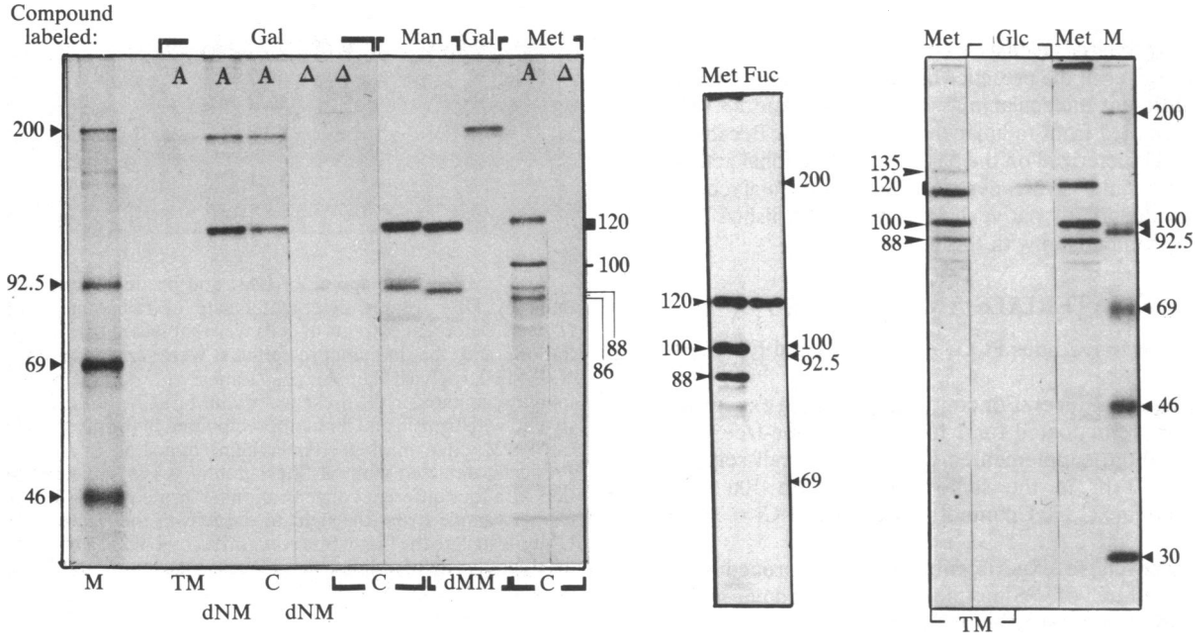

FIG. 3. Immunoprecipitation by DE1 antibody from lysates obtained from sugar-labeled or methionine-labeled EC cells. (*Left*) PCC4 Aza R1 cells were labeled with tritiated mannose (Man) or galactose (Gal) or [^{35}S]methionine (Met) in the absence (control, C) or presence of glycosylation inhibitors: tunicamycin (TM), 1-deoxynojirimycin (dNM) or 1-deoxymannojirimycin (dMM). Lysis of labeled cells was performed in a Triton X 114-containing buffer. The unseparated lysate or the aqueous (lanes A) and detergent (lanes Δ) phase obtained after a fractionation procedure were submitted to immunoprecipitation. $NaDodSO_4$/PAGE was carried out on a linear gradient of 7.5%–15% acrylamide. Size markers in kDa are shown in lane M. A product with an electrophoretic mobility slightly slower than that of the 88-kDa polypeptide was only detected in sugar-labeling experiments. This product partitions into the detergent phase of a Triton X 114 lysate and is affected by the presence of glycosylation inhibitors. (*Center*) PCC4 Aza R1 cells labeled with tritiated fucose (Fuc) and F9 cells labeled with [^{35}S]methionine (Met) were lysed and submitted to immunoprecipitation. $NaDodSO_4$/PAGE was carried out on a linear gradient of 7.5–15% acrylamide. Sizes are shown in kDa. (*Right*) Detergent extracts of glucosamine (Glc)- and methionine (Met)-labeled F9 cells were subjected to immunoprecipitation, and the immunoprecipitates were analyzed on a 7.5–15% $NaDodSO_4$/polyacrylamide gel. In the presence of tunicamycin (TM), UM is labeled with [^{35}S]methionine but no longer with glucosamine. Size markers in kDa are shown in lane M.

medium supplemented with 10% fetal calf serum and then 1 hr in methionine-free Eagle's normal medium supplemented with 7% fetal calf serum before labeling. All media contained 5 μg of tunicamycin (Sigma) per ml.

Cell Lysates. Cells were washed with cold phosphate-buffered saline and then lysed on the plates in 10 mM Tris, pH 7.4/2 mM $CaCl_2$/5 mM $MgCl_2$/0.5% Nonidet P-40 or Triton X 114. The lysate was centrifuged (15 min in an Eppendorf centrifuge), and the supernatant was kept on ice for immunoprecipitation.

After Triton X 114 extraction, the phase separation protocol of Bordier was used (14). $CaCl_2$ (2 mM) and the protease inhibitors benzamidine (15 μg/ml), pepstatine (1 μg/ml), and antipaine (1 μg/ml) were included in buffers unless otherwise indicated.

Trypsin Digestion. Trypsin digestion was carried out on cell lysates (without protease inhibitors) at a trypsin (Sigma) concentration of 50 μg/ml at 37°C for 1 hr. Digestion was stopped by addition of soybean trypsin inhibitor (Sigma) at 100 μg/ml.

Immunoprecipitation. Immunoprecipitation was carried out as described (15) with a buffer containing 2 mM $CaCl_2$ instead of 5 mM EDTA. The rat monoclonal antibody DE1 anti-UMt (10) was used at a concentration of 8 μg/ml of cell lysate. A rabbit polyclonal antibody was raised against purified UMt (9). Immunization was performed as described (16) by injection into the popliteal lymph nodes. This antibody was used at a 1:200 or 1:500 dilution.

Isolation of Cell Surface UM. The prebinding assay (13, 15) was used with the following modifications. F9 cells labeled in suspension were spun and resuspended in 25 μl of phosphate-buffered saline containing 10 μg of the DE1 antibody. After a 7-min incubation on ice, the cell suspension was diluted with phosphate-buffered saline containing 10% fetal calf serum to a final volume of 500 μl and was layered over a cushion of 500 μl of 0.5 M sucrose in phosphate-buffered saline. Cells were centrifuged through the sucrose cushion (2 min in an Eppendorf centrifuge). The cell pellet was lysed as described previously with an equivalent amount of unlabeled F9 cells. Surface UM and intracellular UM were then isolated as described for HLA antigens (13).

$NaDodSO_4$/PAGE. Sample preparation and $NaDodSO_4$/PAGE were carried out as described (17). Radioactive products (except those used for peptide maps) were detected by fluorography.

Peptide Mapping Experiments. Peptide maps were prepared as described (18). V8 protease (Sigma) was dispensed from a solution (0.2 mg/ml) stored frozen. Over each gel slice was layered 10 μl of 10% sucrose/0.125 M Tris, pH 6.8/0.1% $NaDodSO_4$/1 mM EDTA/0.0001% bromophenol blue containing the appropriate amount of protease. Electrophoresis was carried out on a 12% $NaDodSO_4$/polyacrylamide gel. Digestion took place during the 2 hr of migration of the samples in the stacking gel.

Sucrose Gradient. Gradients were performed as described (19) with sucrose instead of glycerol. Sucrose-containing solutions were prepared in immunoprecipitation buffer.

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was performed as described (20). Samples were applied to the acidic end of gradients prepared with the following Ampholine concentrations: 1.6% of pH 3.5–10 and 0.4% of pH 5–7. Electrophoresis took place during 7 hr at 350 V. Alternatively, samples were applied to the basic end of gradients prepared with Ampholine concentrations 0.75% of pH 3.5–5, 0.65% of pH 3.5–10, and 0.6% of pH 4–6, and electrophoresis took place for 12 hr at 400 V and then for 1 hr at 800 V.

Immunoreplicas. Immunoreplicas were performed as described (21) by using anti-UMt rabbit polyclonal antibody at a 1:750 dilution and peroxidase-conjugated anti-rabbit antiserum (Bio-Sys) at a concentration of 2 μg/ml.

RESULTS

Structural Relationship Between the Different Molecules Immunoprecipitated by DE1 in a Detergent Lysate of F9 Cells. In a detergent lysate of continuously labeled F9 cells, the rat monoclonal antibody DE1 recognized in the presence of calcium three products having molecular masses of 120, 100, and 88 kDa (Fig. 1). Immunoprecipitation of these products with DE1 or rabbit polyclonal antibodies raised against purified UMt could be competitively inhibited with an excess of purified UMt (not shown). In addition, DE1 recognized a 135-kDa doublet in a detergent lysate of pulse-labeled F9 cells (Fig. 2). The relationship between these polypeptides was investigated by peptide map analysis by the method of Cleveland using V8 protease (18) (Figs. 1 and 2). The 120-kDa polypeptide was found not to be clearly related to the 100- and 88-kDa polypeptides, while the latter two themselves did not show any strong resemblance either. In contrast, the 135- and 120-kDa polypeptides are structurally related (Fig. 2).

Structural Relationship Between UMt and the Different Molecules Immunoprecipitated by DE1. When compared with the major tryptic fragment of uvomorulin (UMt_1), the 120-kDa polypeptide showed many peptides in common (Fig. 1). Therefore, it is concluded that the 120-kDa polypeptide, which will be referred as UM, is structurally related to UMt, while the 100- and 88-kDa polypeptides are not, nor are the latter two related to each other.

Comparison of Products Immunoprecipitated by DE1 in Different Cell Types. Immunoprecipitation carried out with DE1 showed that cell lines PCC4 Aza R1 and F9 differ in the

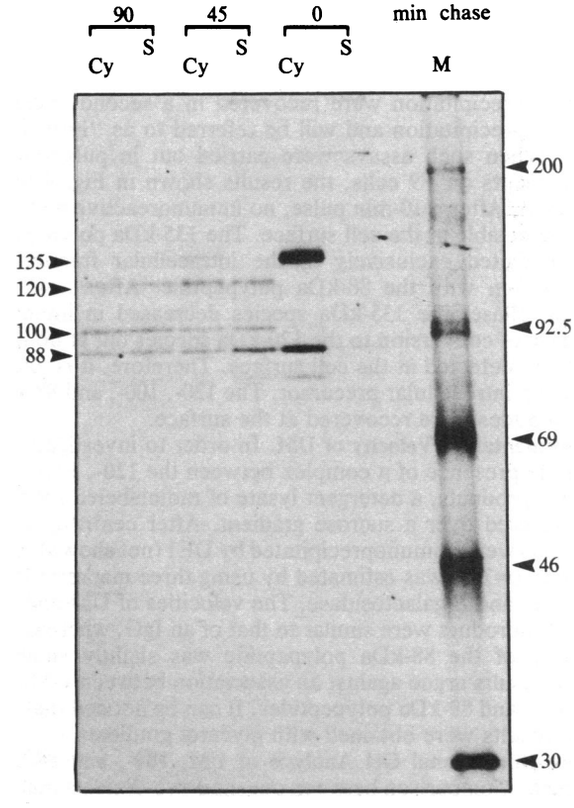

FIG. 4. Isolation of cell surface UM. Cell surface (S) and intracellular (Cy) UM were isolated as described from F9 cells pulse-labeled for 10 min and chased for 0, 45, or 90 min. Size markers in kDa are shown in lane M. $NaDodSO_4$/PAGE was carried out on a linear gradient of 7.5–15% acrylamide.

amount of 88-kDa product recovered. An additional polypeptide (86 kDa) was recovered in a lysate of PCC4 Aza R1 cells (Fig. 2). Cleveland map analysis of the products recognized by DE1 in the two cell lines shows (Fig. 2) the presence of the same products of 120, 100, and 88 kDa in both cell lines. The additional 86-kDa polypeptide could be a degradation product of the 88-kDa molecule. Mouse EC lines PCC3 and LT-1 (22), the cytotrophoblastoma cell line TDM-1 (22), and the dog epithelial cell line MDCK (23), as well as mouse blastocysts, were assayed for the presence of UM, 100-, and 88-kDa products. In all cases, the three different products were immunoprecipitated together. In mouse fibroblastic cells (3T3 and 3T6) known for the absence of detectable UM (3), none of them was found (not shown).

Sugars Are Not Responsible for Differences in Cleveland Maps. Only UM, but not the 100- and 88-kDa products, was affected by the presence of tunicamycin during labeling procedures (Fig. 3 *Right*). Thus, UM appears to carry N-linked oligosaccharides. Cleveland maps were prepared from tunicamycin-treated UM in order to estimate differences in peptide mobility that might be due to the presence of N-linked sugars. Although the mobility of several peptides was affected by tunicamycin treatment, it was still concluded that the 120-, 100-, and 88-kDa products are not related to each other (data not shown).

Sugar Labeling of UM. UM, but not the 100- and 88-kDa products, was labeled with various sugars (mannose, galactose, fucose, and glucosamine) (Fig. 3). UM was no longer labeled with galactose or glucosamine in the presence of tunicamycin (Fig. 3 *Left* and *Right*). These results indicate that UM probably carries only N-linked oligosaccharides, some of them being of the complex type. The 100- and 88-kDa products are unlikely to be glycosylated.

Cell Surface Expression of UM. Surface expression of UM was examined by performing binding assays with antibodies to intact cells, followed by removal of excess antibody and recovery of immune complexes (surface fraction) from a detergent lysate. Molecules not isolated in this first round of immunoprecipitation were recovered in a second cycle of immunoprecipitation and will be referred to as "intracellular." When such assays were carried out in pulse–chase experiments on F9 cells, the results shown in Fig. 4 were obtained. After a 10-min pulse, no immunoreactive material was detectable at the cell surface. The 135-kDa polypeptide was detected exclusively in the intracellular fraction in conjunction with the 88-kDa polypeptide. After a 45- or 90-min chase, the 135-kDa species decreased in intensity because of conversion to the 120-kDa species but at no time could be detected at the cell surface. Therefore, it is exclusively an intracellular precursor. The 120-, 100-, and 88-kDa polypeptides were recovered at the surface.

Sedimentation Velocity of UM. In order to investigate the possible presence of a complex between the 120-, 100-, and 88-kDa products, a detergent lysate of radiolabeled F9 cells was layered over a sucrose gradient. After centrifugation, fractions were immunoprecipitated by DE1 (not shown). UM velocity (≈7 S) was estimated by using three markers: IgG, catalase, and β-galactosidase. The velocities of UM and the 100-kDa product were similar to that of an IgG, whereas the velocity of the 88-kDa polypeptide was slightly smaller. These results argue against an association between UM and the 100-, and 88-kDa polypeptides. It can be noticed that the same results were obtained with glycerol gradients.

Two-Dimensional Gel Analysis of UM, 100-, and 88-kDa Products. Comparison between one- and two-dimensional gel electrophoresis of immunoprecipitated UM led to the conclusion that the 100- and 88-kDa products have a more basic isoelectric point than that of UM, which is very acidic (pH 4). The high number of spots observed in a two-dimensional gel analysis suggests that a degradation of UM occurs during denaturation of the samples in urea or that bands identified in one-dimensional gels are heterogeneous (Fig. 5). Immunoreplicas of one- or two-dimensional gels showed that only UM and its putative degradation products (having similar acidic isoelectric point) are recognized by anti-UMt polyclonal antibodies (Fig. 5).

DISCUSSION

By immunoprecipitation with anti-UMt antibodies (either monoclonal or polyclonal), a series of products (120, 100, and 88 kDa) are recovered from different cell types. This result is specific because immunoprecipitation of these three polypeptides (*i*) by DE1 requires calcium and (*ii*) by DE1 or rabbit polyclonal antibodies raised against purified UMt can be competitively inhibited with an excess of purified UMt. The polypeptides immunoprecipitated by DE1 are unlikely to result from a proteolytic degradation of the heaviest product identified (120 kDa) as was hypothesized (2–4). Peptide map comparisons presented here establish the relationship of the 120-kDa polypeptide with UMt and demonstrate the existence of a 135-kDa intracellular biosynthetic precursor of UM. The generation of the 100-kDa and 88-kDa polypeptides by proteolysis of UM can be excluded on the basis of peptide maps. This means that the 100-kDa and 88-kDa polypeptides are unlikely to represent different postranslational processing forms of an UM precursor, even though at least the 100-kDa polypeptide becomes detectable only after a certain period of chase (see Fig. 4).

A number of observations were consistent with the hypothesis of a complex between UM and the 100- and 88-kDa polypeptides. The three polypeptides are coimmunoprecipitated, but UM only is recognized in immunoreplicas. All three products are detected at the cell surface, although the 100- and 88-kDa polypeptides are devoid of detectable sugars. The three products are coexpressed in the different cell types examined. However, determination of the sedimentation constant by centrifugation does not support this possibility.

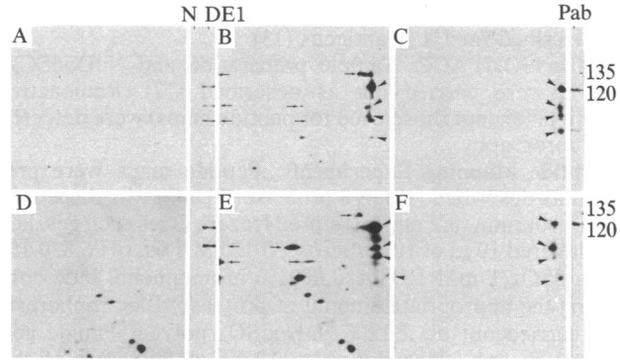

FIG. 5. Two-dimensional gel electrophoresis of UM. Membranes prepared as described (2) from F9 cells (*C* and *F*) and material immunoprecipitated by DE1 (*B* and *E*) or by normal rabbit serum (*A* and *D*) from F9 cells labeled for 4 hr with [^{35}S]methionine were analyzed in two-dimensional gel electrophoresis. Samples were applied either to the acidic end of the Ampholine gradient (*A*, *B*, and *C*) or to the basic end (*D*, *E*, and *F*) as described. Two-dimensional gels (*A*, *B*, *D*, and *E*) or immunoreplicas of two-dimensional gels (*C* and *F*) are shown in parallel with a $NaDodSO_4$/PAGE of the same samples. Lanes: N, immunoprecipitation by normal rabbit serum; DE1, immunoprecipitation by DE1; Pab, membranes prepared from F9 cells analyzed by immunoreplica with anti-UMt polyclonal antibody. Arrows indicate the 135-, 100-, and 88-kDa products. Arrowheads indicate the mature form of UM (120 kDa) and three smaller products having the same isoelectric point that are both immunoprecipitated and recognized in immunoreplicas. The acidic end of the gels are oriented towards the right of the figure. Sizes are shown in kDa.

Results presented here can best be explained by assuming the presence of shared epitopes on the different polypeptides. The 100- and 88-kDa polypeptides are not recognized in immunoreplicas. An explanation for this observation would be that suitable renaturation of the epitopes recognized does not occur on nitrocellulose filters. Sensitivity to denaturation of the antigens recognized by anti-UMt antibodies also was observed in immunoprecipitation experiments. UM only (120 kDa) is immunoprecipitated from cells boiled in the presence of $NaDodSO_4$ as described for the isolation of L-CAM (liver cell adhesion molecule) (24).

Biochemical studies of UM or UM-like molecules have been carried out in different species (3, 4, 6, 24). Current data available do not exclude that several antibodies recognize families of products comparable to what is described here.

The functional role of the 100- and 88-kDa products requires further study. The presence at the cell surface of those two products is consistent with a putative role in cell–cell interactions. The absence of carbohydrates on these polypeptides is unusual for proteins exposed at the cell surface if not themselves associated with glycosylated species. However, a small number of sugar residues could have remained undetected in *in vivo* labeling experiments with radioactive sugars but might be revealed using more sensitive techniques.

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